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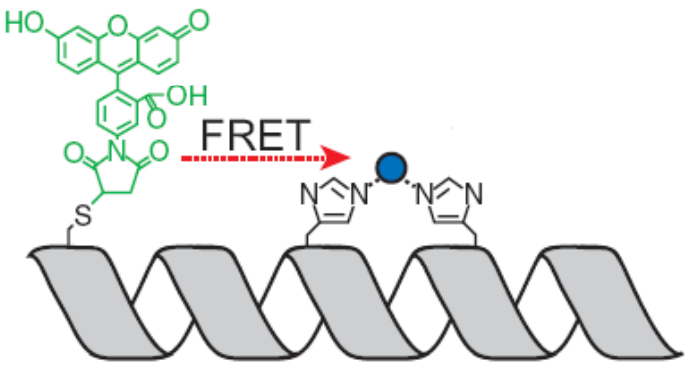
Transition metal ion FRET



Mapping the structure and conformational movements of proteins with transition metal ion FRET

Justin W Taraska[1], Michael C Puljung[1], Nelson B Olivier[2], Galen E Flynn[1] & William N Zagotta[1]

Visualizing conformational dynamics in proteins has been difficult, and the atomic-scale motions responsible for the behavior of most allosteric proteins are unknown. Here we report that fluorescence resonance energy transfer (FRET) between a small fluorescent dye and a nickel ion bound to a dihistidine motif can be used to monitor small structural rearrangements in proteins. This method provides several key advantages over classical FRET, including the ability to measure the dynamics of close-range interactions, the use of small probes with short linkers, a low orientation dependence, and the ability to add and remove unique tunable acceptors. We used this 'transition metal ion FRET' approach along with X-ray crystallography to determine the structural changes of the gating ring of the mouse hyperpolarization-activated cyclic nucleotide–regulated ion channel HCN2. Our results suggest a general model for the conformational switch in the cyclic nucleotide–binding site of cyclic nucleotide–regulated ion channels.

In allosteric proteins, the binding of a ligand stabilizes conformational rearrangements, resulting in a switch between inactive and active states. These movements can propagate long distances to affect activity tens of angstroms from the ligand-binding site. In HCN channels, the direct binding of cAMP to a cytoplasmic ligand-binding domain stabilizes the opening conformational changes in the channel pore located more than 50 Å away[1]. However, as in most allosteric proteins, the direction, magnitude and nature of conformational changes in the channel are unknown.

Fluorescence resonance energy transfer (FRET), in which light energy absorbed by a donor is transferred to a nearby acceptor, is a powerful tool for measuring changes in molecular distances[2]. The efficiency of FRET falls off with the sixth power of the distance between the two molecules, making FRET exquisitely sensitive to changes in distance. However, as a consequence of this steep distance-dependence, FRET can be used to measure distances effectively only in a narrow window around R_0, the distance at which FRET efficiency is 50% (ref. 2). Classical FRET methods are not always well-suited to study intramolecular movements in proteins. This is chiefly due to the long R_0 values of most fluorescent dye or protein FRET pairs (30–60 Å), their large sizes (15–30 Å) and their long flexible attachment linkers (10–15 Å)[3,4]. Combined with the dependence of the FRET on the relative orientation of the fluorophores, these properties can complicate the interpretation of FRET results. Whereas lanthanide resonance energy transfer methods have less orientation dependence than FRET, they still use large chelating groups and dyes with long linkers, have similarly long R_0 values (~30–100 Å) and have the added complication of very long excited state lifetimes[3,5].

What is needed to map conformational rearrangements in proteins is a technique that can work over shorter distances than classical FRET. We developed such a method, called transition metal ion FRET, which has shorter R_0 values (about 10 Å), uses small dyes with short linkers and is not sensitive to the orientation problems usually associated with FRET. Unlike NMR spectroscopy, electron paramagnetic resonance and crystallography, transition metal ion FRET could theoretically be applied to proteins of any size, in membranes, on extracellular domains of proteins in living cells and potentially on single molecules. It can be used to measure the kinetics of the conformational change in real time and can be paired with other functional measurements. Here we used this method along with X-ray crystallography to explore the conformational motions of the C-terminal region of HCN2 during ligand activation. Our results demonstrate the ability of transition metal ion FRET to reveal structural rearrangements in proteins and provide a model for allosteric movements of HCN2.

RESULTS

Transition metal ion FRET

Our fluorescence method relies on two previous observations. First, transition metal ions such as Ni^{2+} and Cu^{2+} are colored, and their weak absorbance values overlap with the emission spectra of fluorescent dyes including fluorescein (Fig. 1a). Thus, Ni^{2+} and Cu^{2+} can be used as short-range FRET acceptors[6,7]. Indeed, the R_0 value for Ni^{2+} and fluorescein (12 Å) is four times shorter than standard FRET pairs such as CFP and YFP (50 Å) or fluorescein and rhodamine (54 Å)[8,9]. The R_0 value between Cu^{2+} and fluorescein is longer (16 Å), allowing transition metal ion FRET to be tuned to different distance ranges. Second, a metal-binding site can be engineered into proteins with minimal perturbation to the backbone by introducing two histidines spaced one turn away on an α-helix[10,11] (Fig. 1b). We reasoned that by engineering a metal

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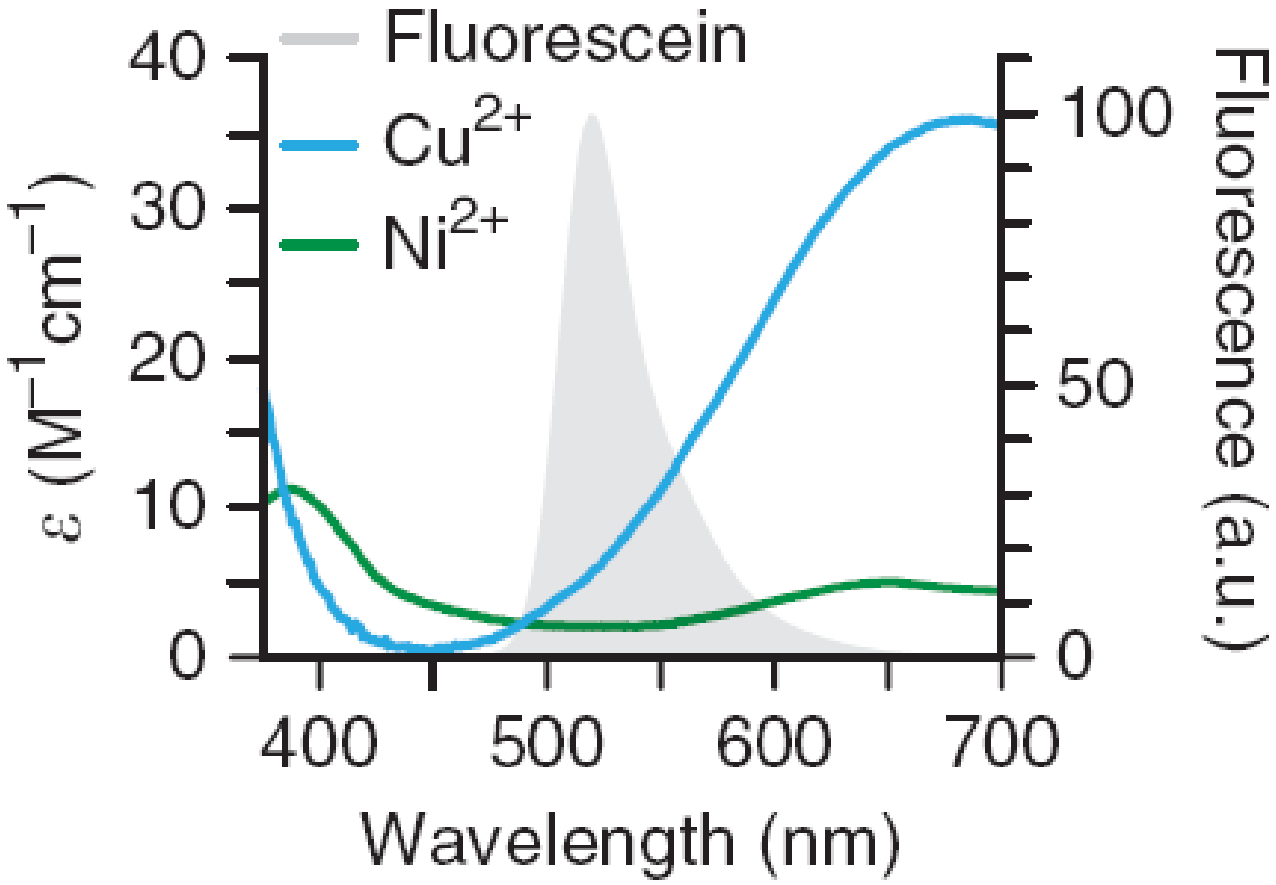
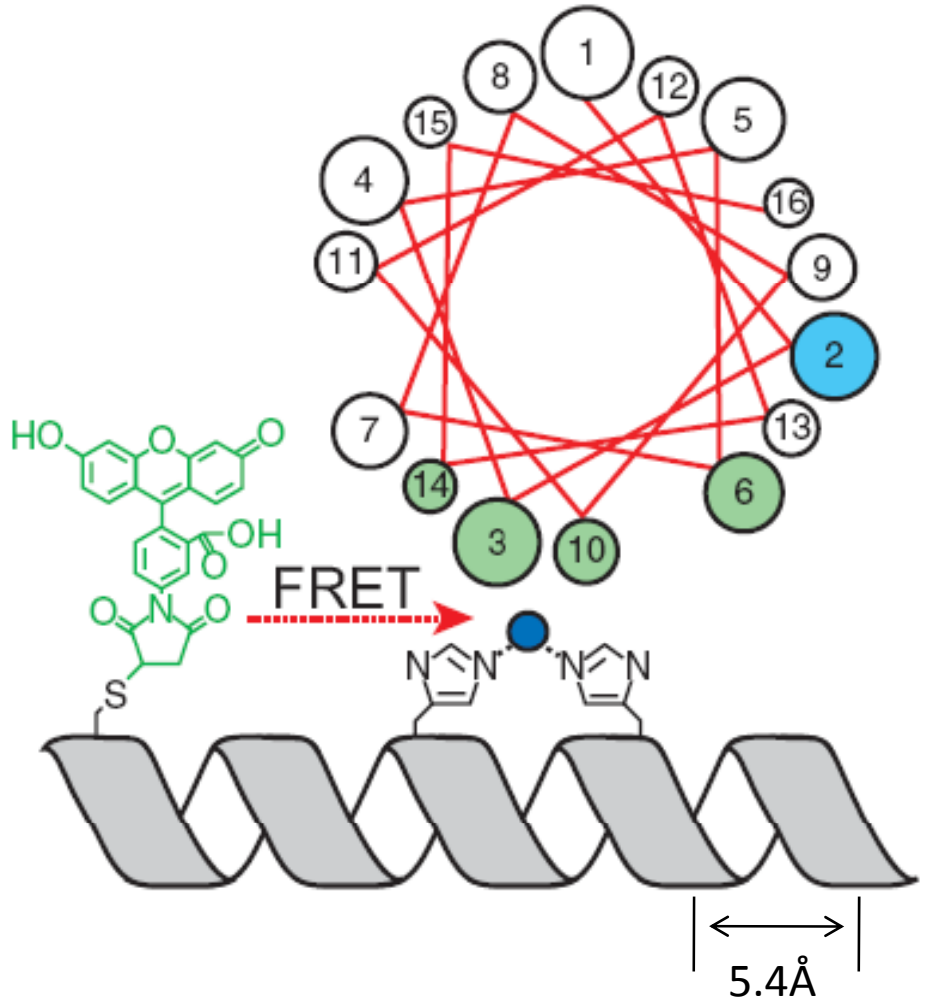
FRET between Fluorescein and transition metal ion

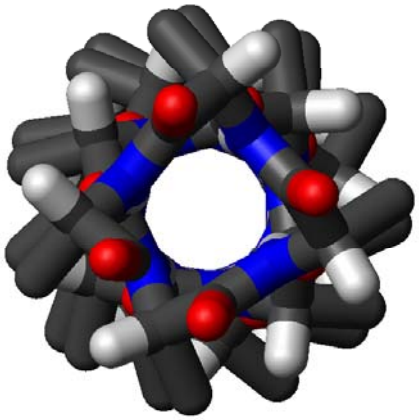


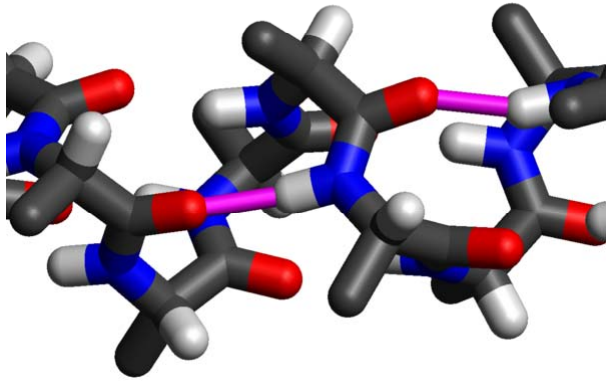
$$E = \frac{F_A}{F_{total}} = \frac{F_{tot} - F_{metal}}{F_{tot}} = 1 - \frac{F_{metal}}{F_{tot}}$$

R_0 of
Ni^{2+}-fluorescein : 1.2 nm
Cu^{2+}-fluorescein : 1.6 nm

Model α-helix peptide



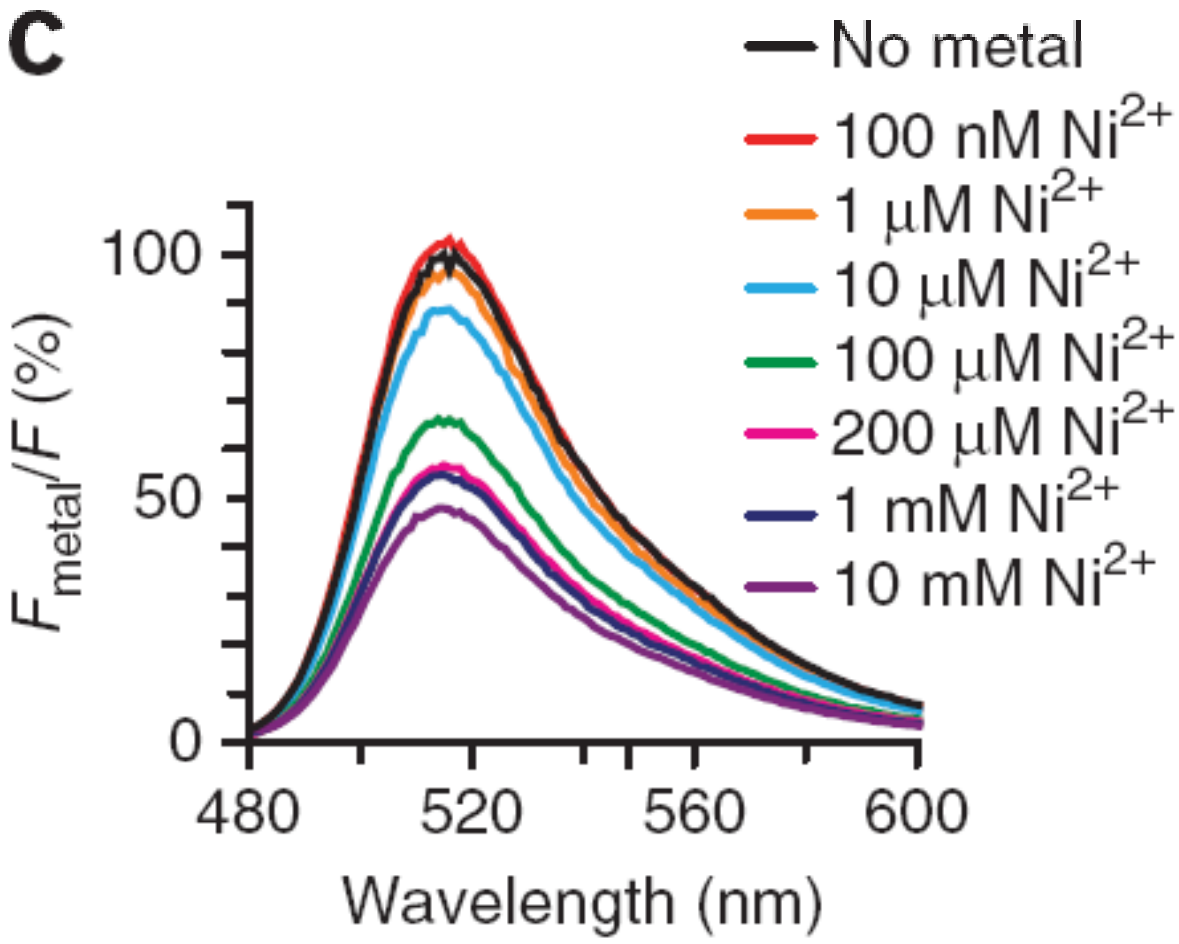




ACAAKAAAKAAAAAKA : C2
ACHAKHAAKAAAAAKA : C2H3H6
ACAAKHAAKHAAAAKA : C2H6H10
ACAAKAAAKHAAAHKA : C2H10H14

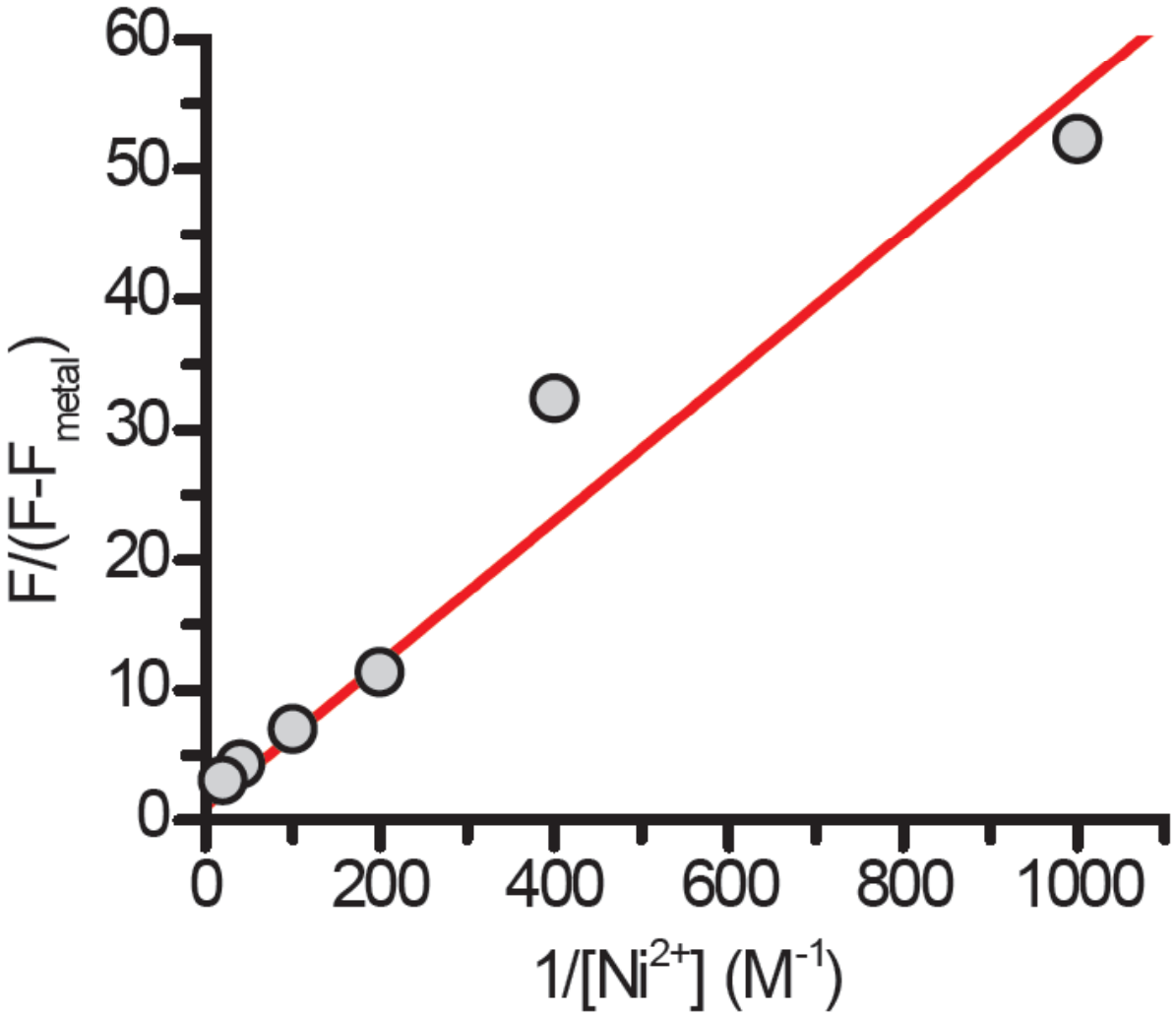
By Increasing Ni^{2+} ions……

ACHAKHAAKAAAAAKA : C2H3H6

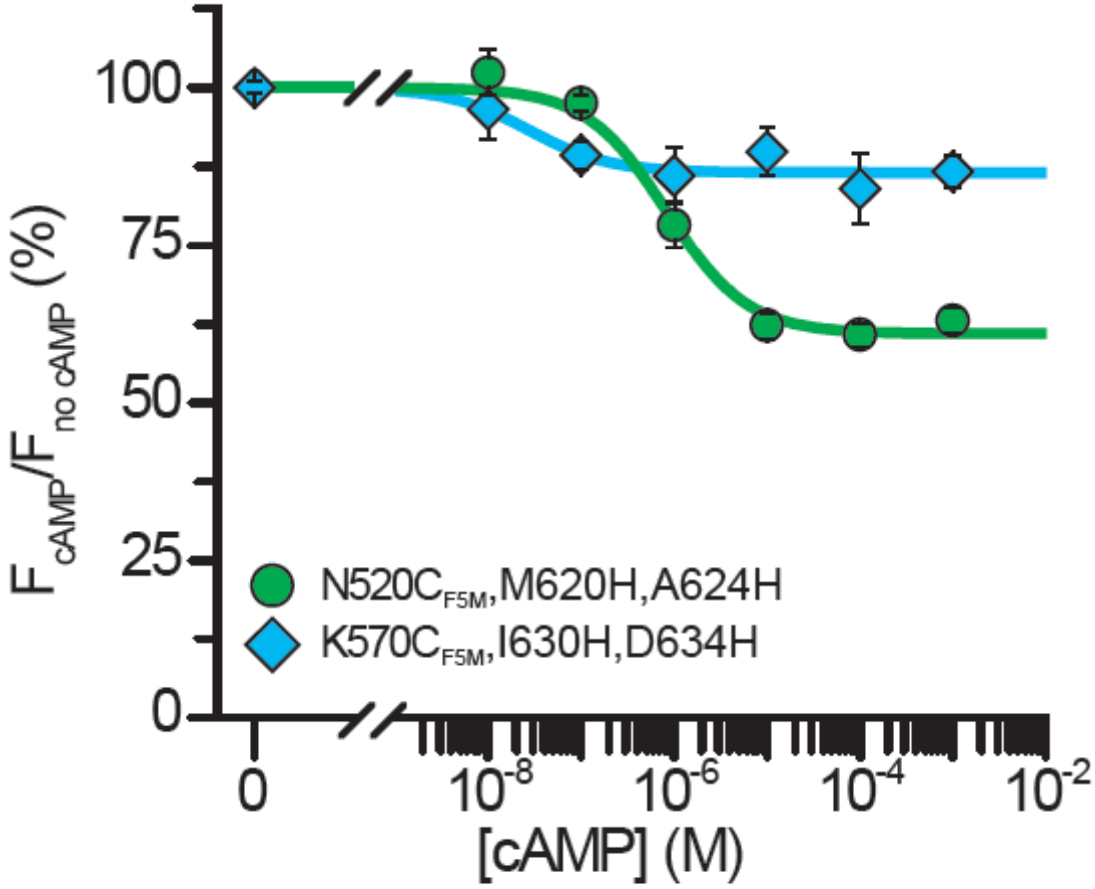


Collisional Quenching

ACAAKAAAKAAAAAKA : C2H3H6

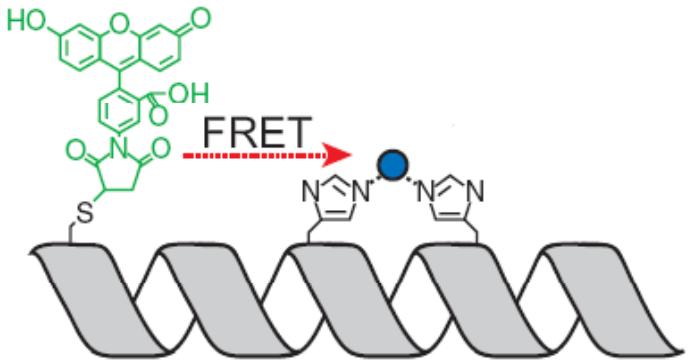


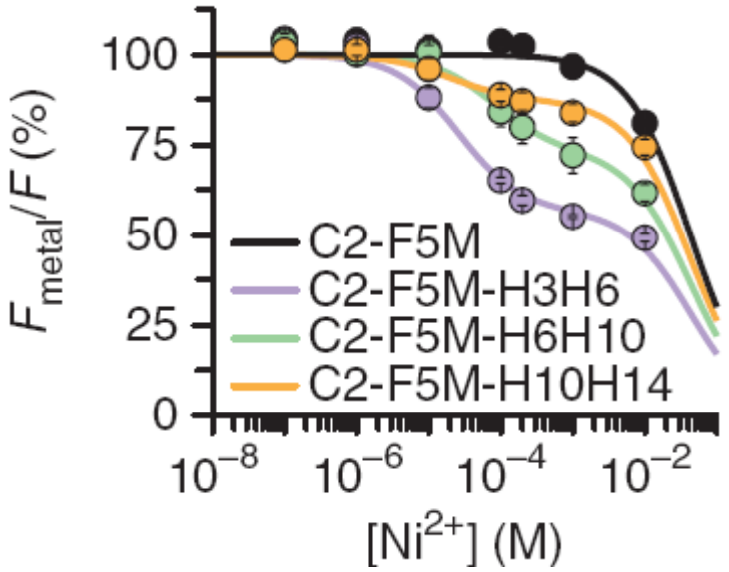
Collisional Quenching



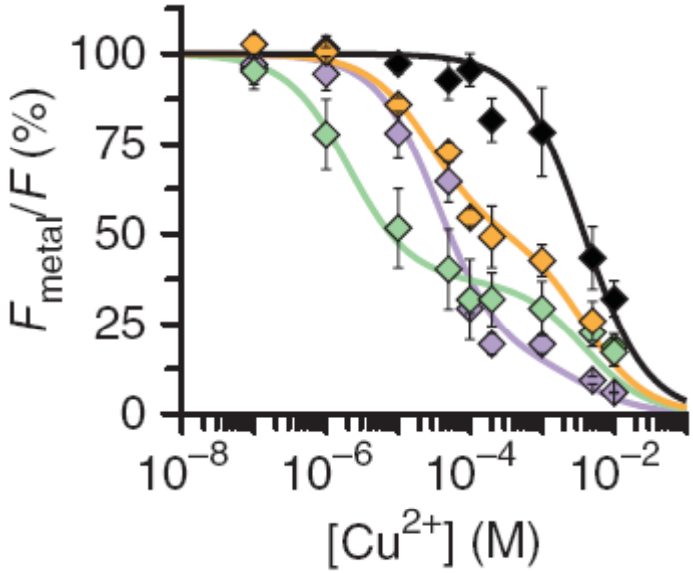
Supplementary Figure 2. cAMP affinity of HCN2 constructs. Fluorescence of $N520C_{F5M}$,M620H,A642H (green) and $K570C_{F5M}$,I630H,D634H (blue) in the presence of 100 μM Ni^{2+} and increasing concentrations of cAMP. All data are normalized to the fluorescence before the addition of cAMP. The Kd of cAMP was 920 nM ± 240 nM for $N520C_{F5M}$,M620H,A642H and 97 nM ± 67 nM for $K570C_{F5M}$,I630H,D634H.

By Increasing the distances

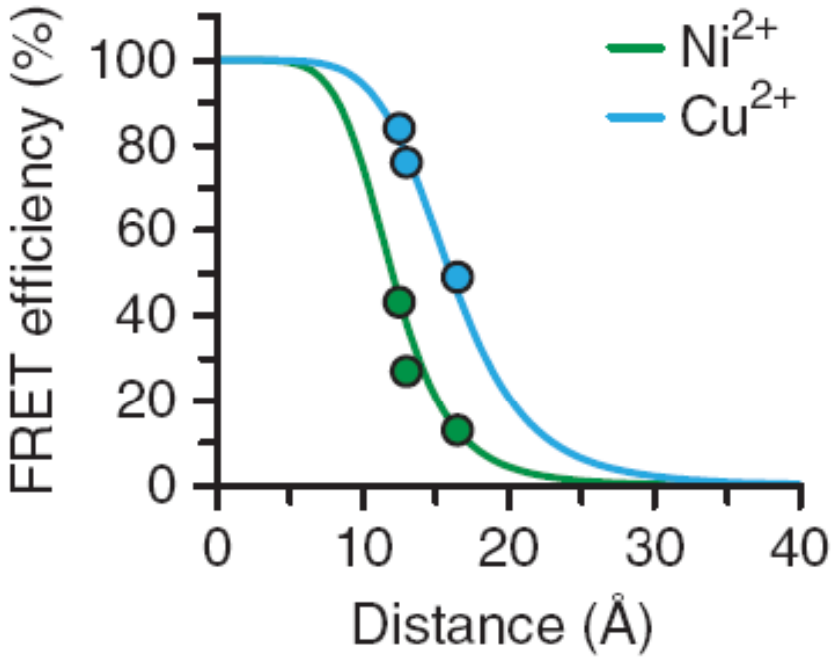


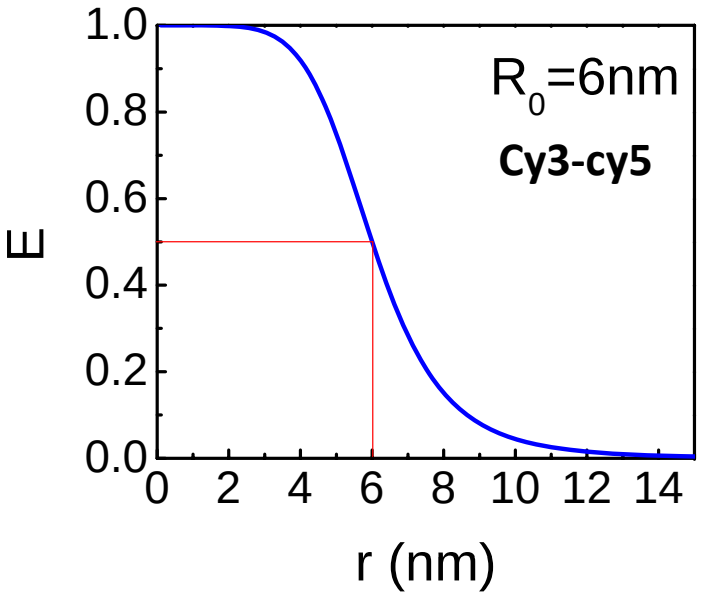


ACAAKAAAKAAAAAKA : C2
ACHAKHAAKAAAAAKA : C2H3H6
ACAAKHAAKHAAAAKA : C2H6H10
ACAAKAAAKHAAAHKA : C2H10H14

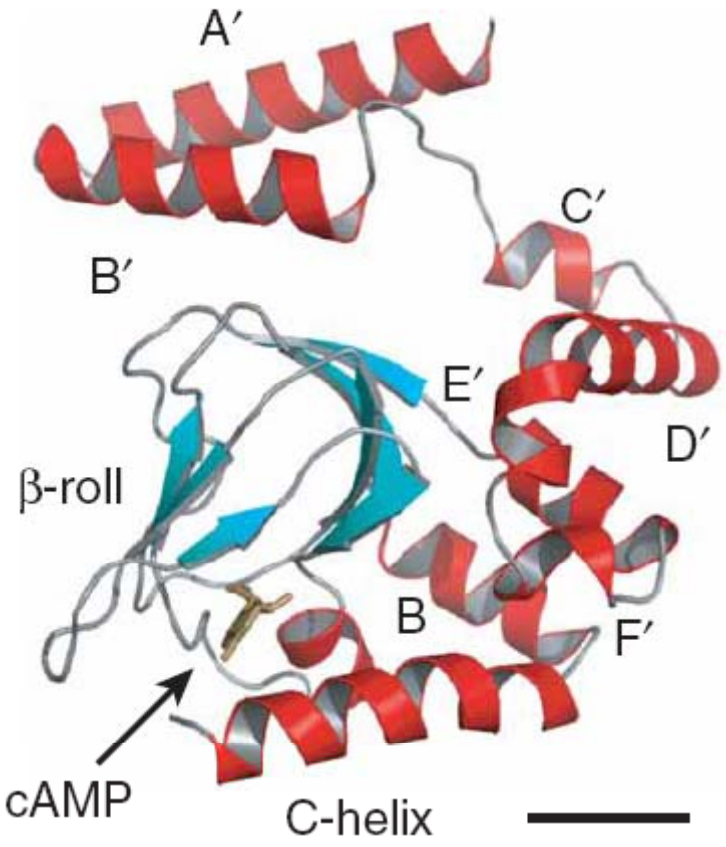


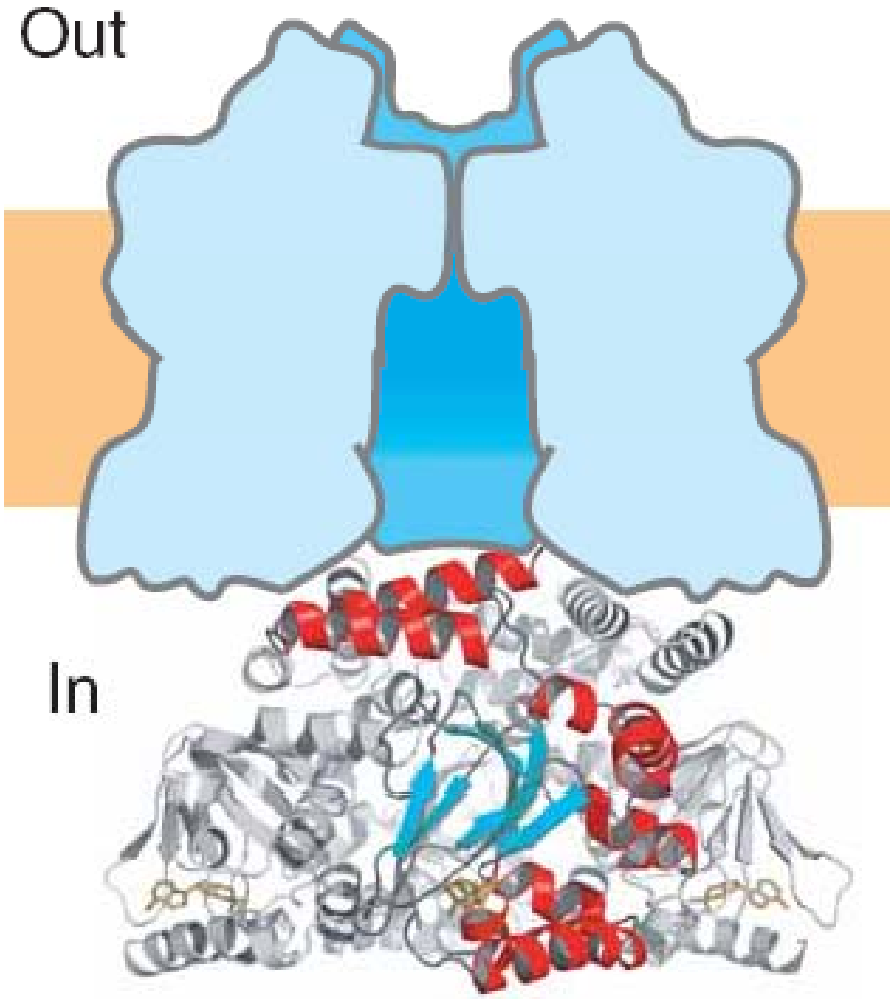
FRET curves


FRET efficiency (%)
100
80
60
40
20
0
0
10
20
30
40
Distance (Å)
Ni2+
Cu2+

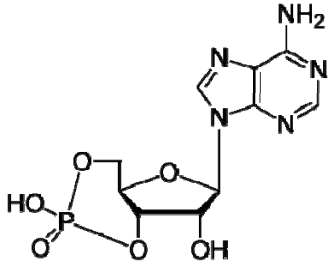

E
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
r (nm)
R0=6nm
Cy3-cy5

Cysteineless HCN2 C-terminal fragment

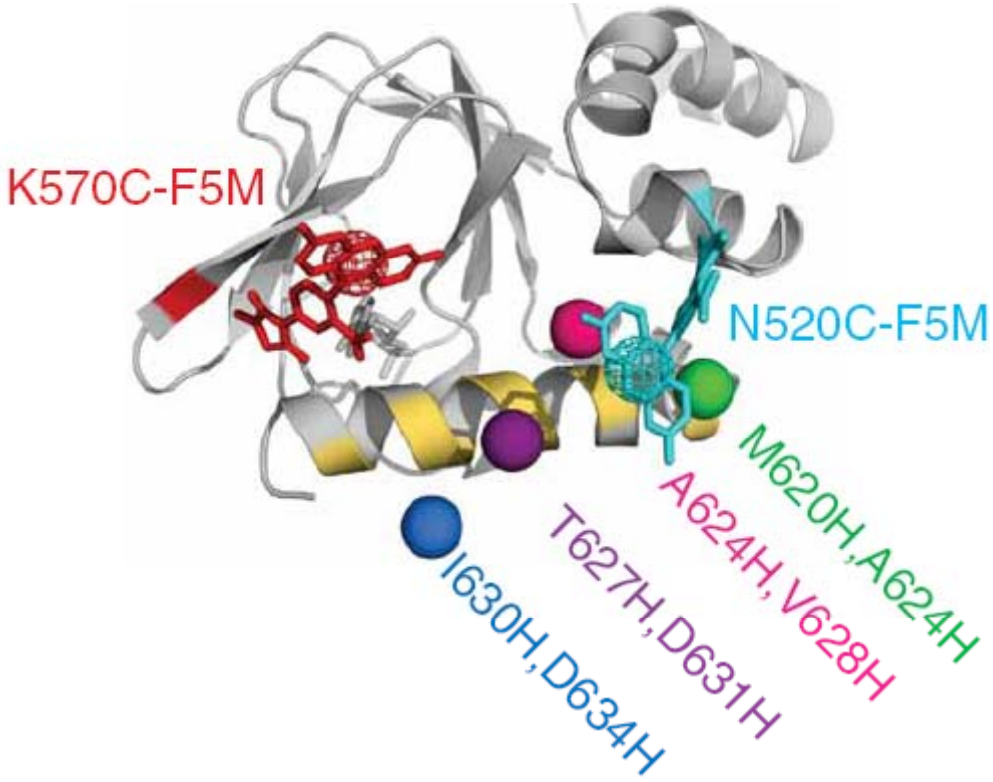


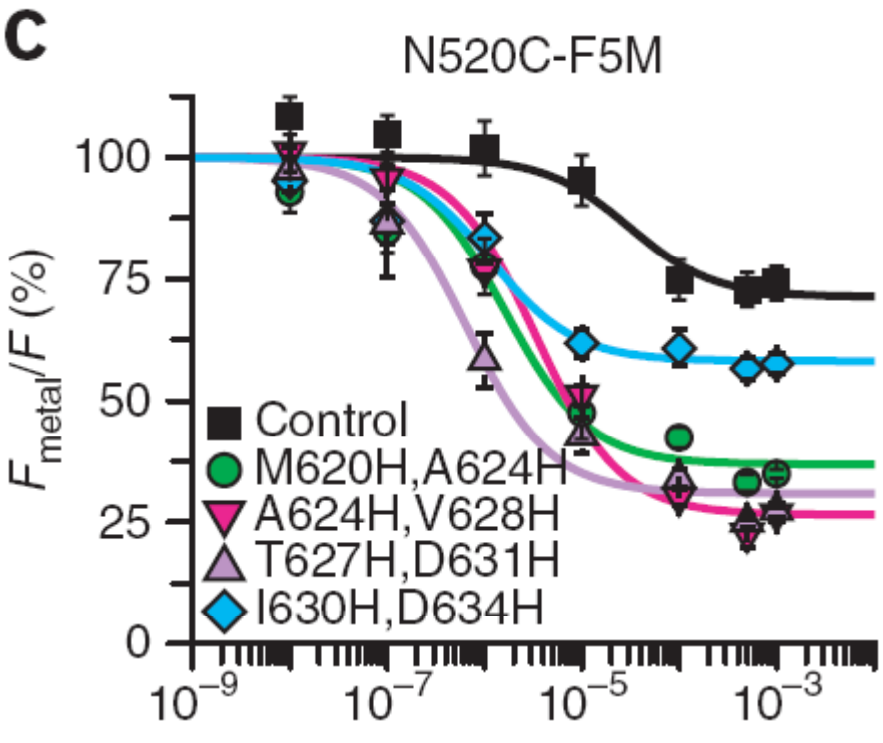


CNBD : cyclic nucleotide-binding domain

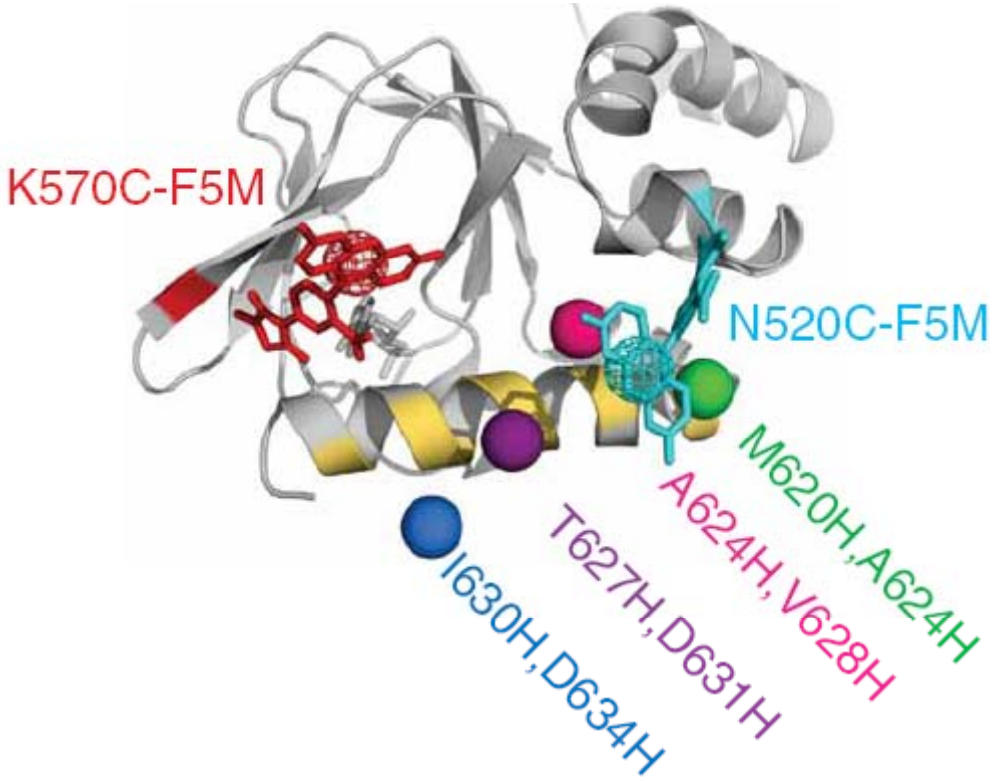


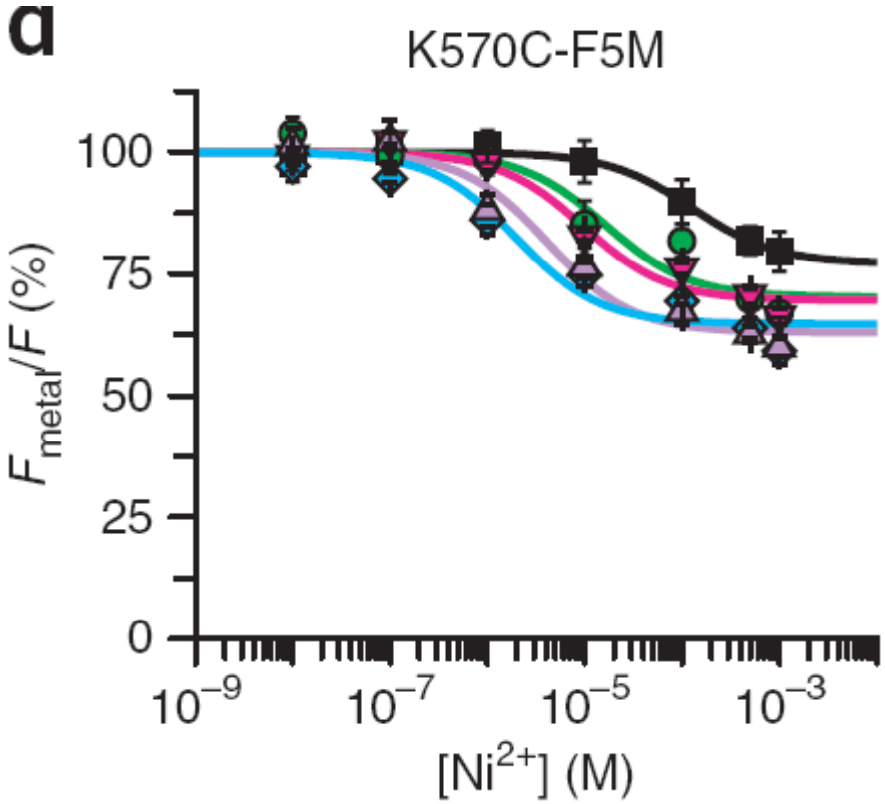
N520C-F5M


K570C-F5M
N520C-F5M
M620H,A624H
A624H,V628H
T627H,D631H
I630H,D634H

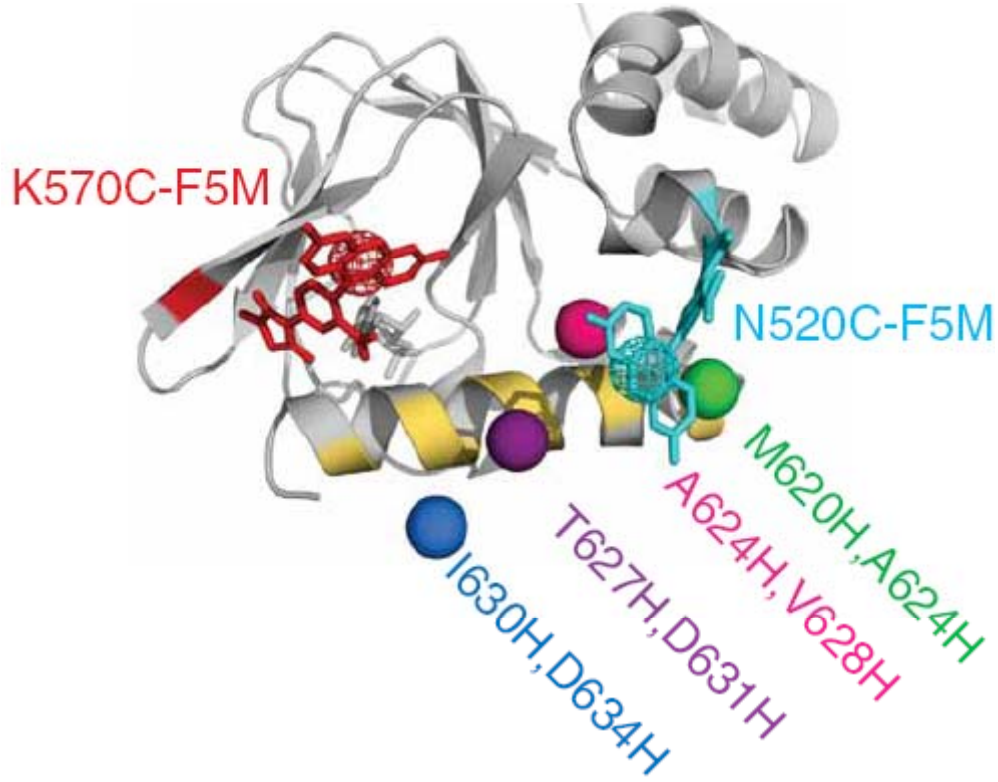

c
N520C-F5M
F_{metal}/F (%)
100
75
50
25
0
10^{-9}
10^{-7}
10^{-5}
10^{-3}
Control
M620H,A624H
A624H,V628H
T627H,D631H
I630H,D634H

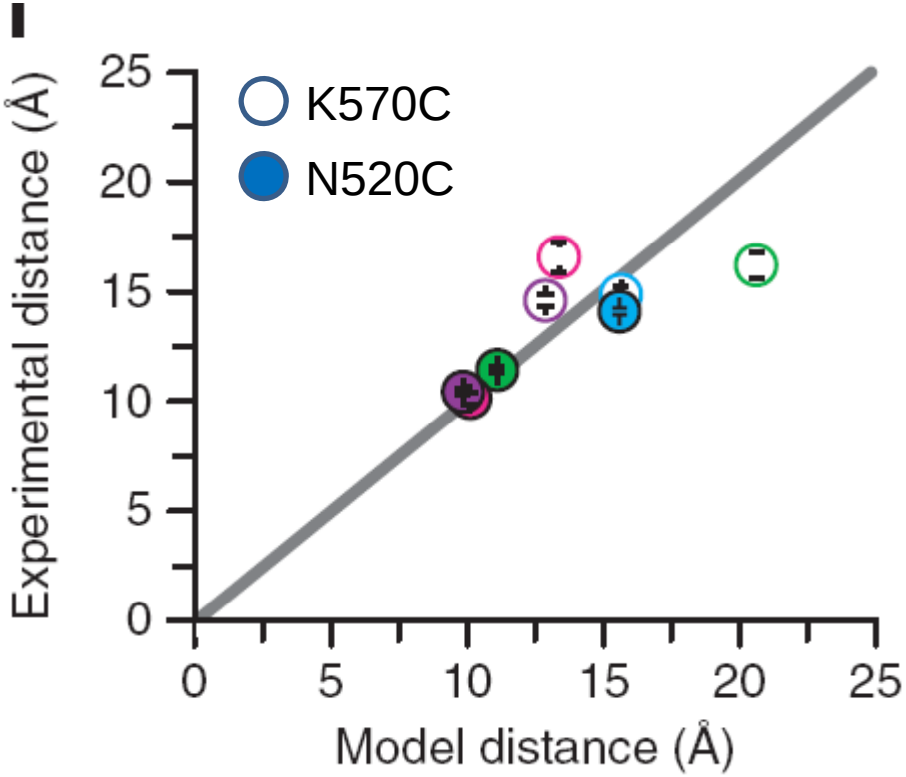
K570C-F5M


K570C-F5M
N520C-F5M
M620H,A624H
A624H,V628H
T627H,D631H
I630H,D634H


a
K570C-F5M
100
75
50
25
0
F_{metal}/F (%)
10^{-9}
10^{-7}
10^{-5}
10^{-3}
[Ni^{2+}] (M)

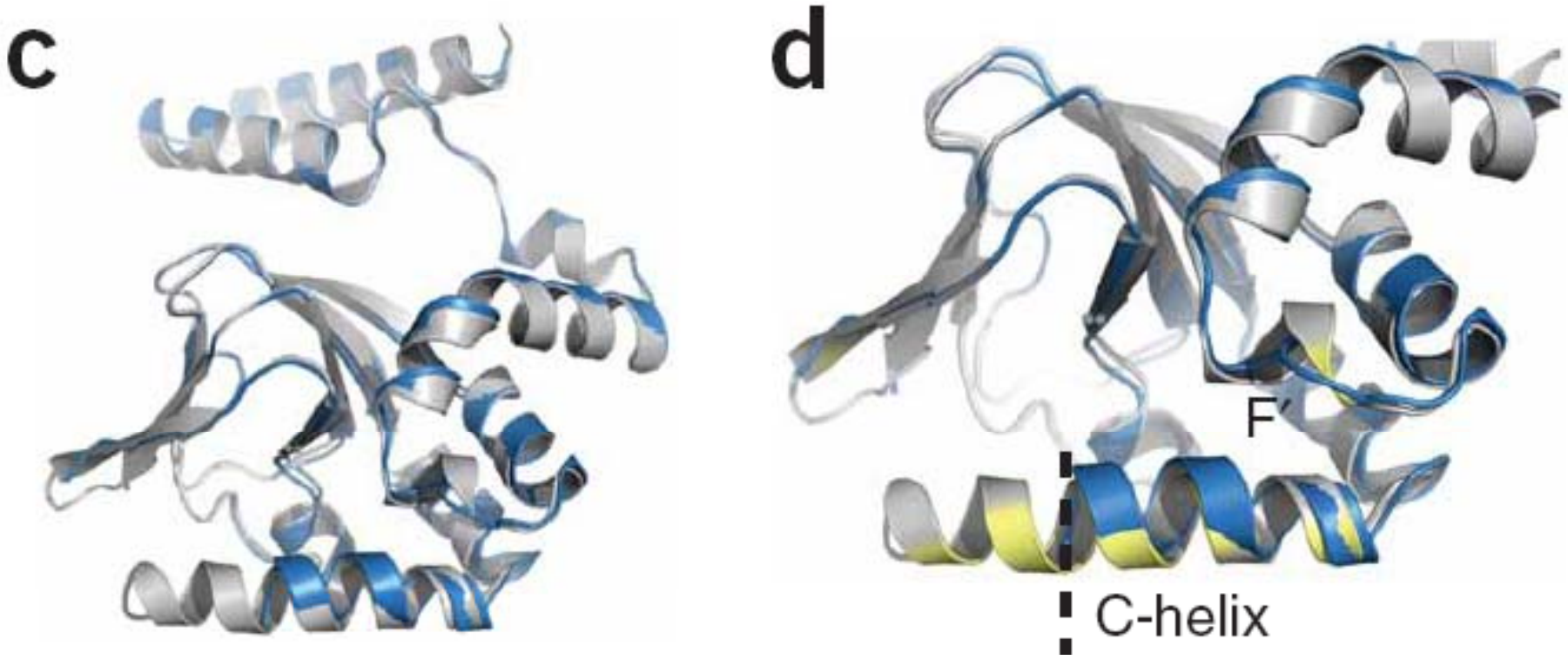
Distance measured



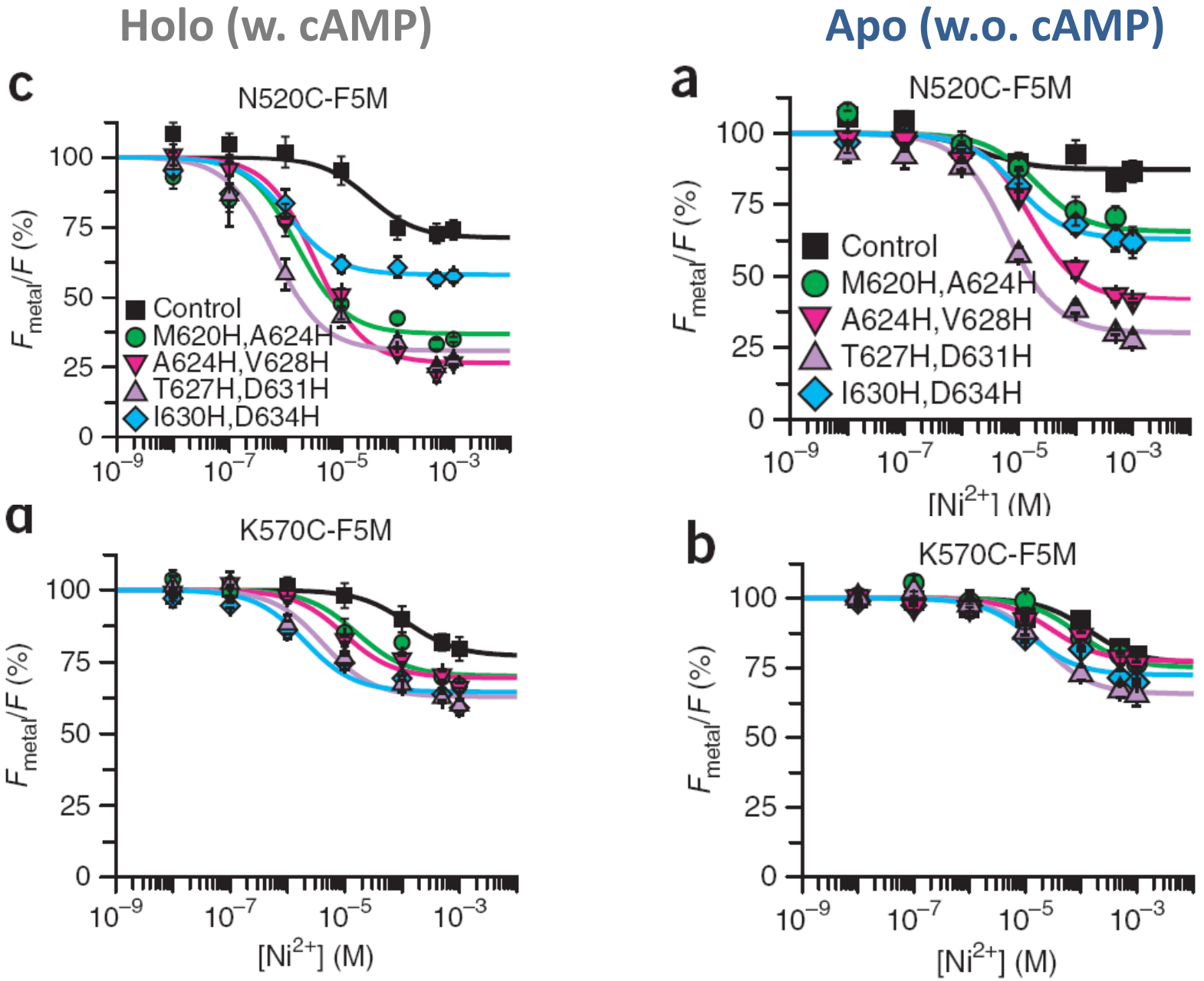


Overay of

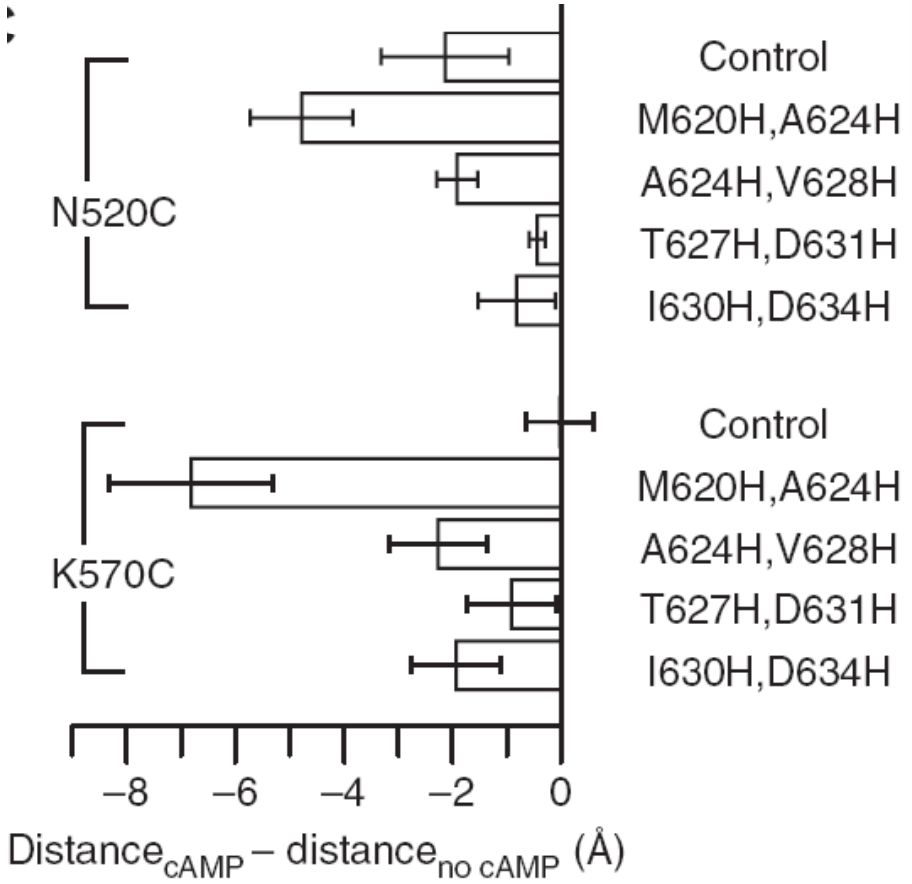
The apo (w.o. cAMP) and holo (w. cAMP) state HCN2

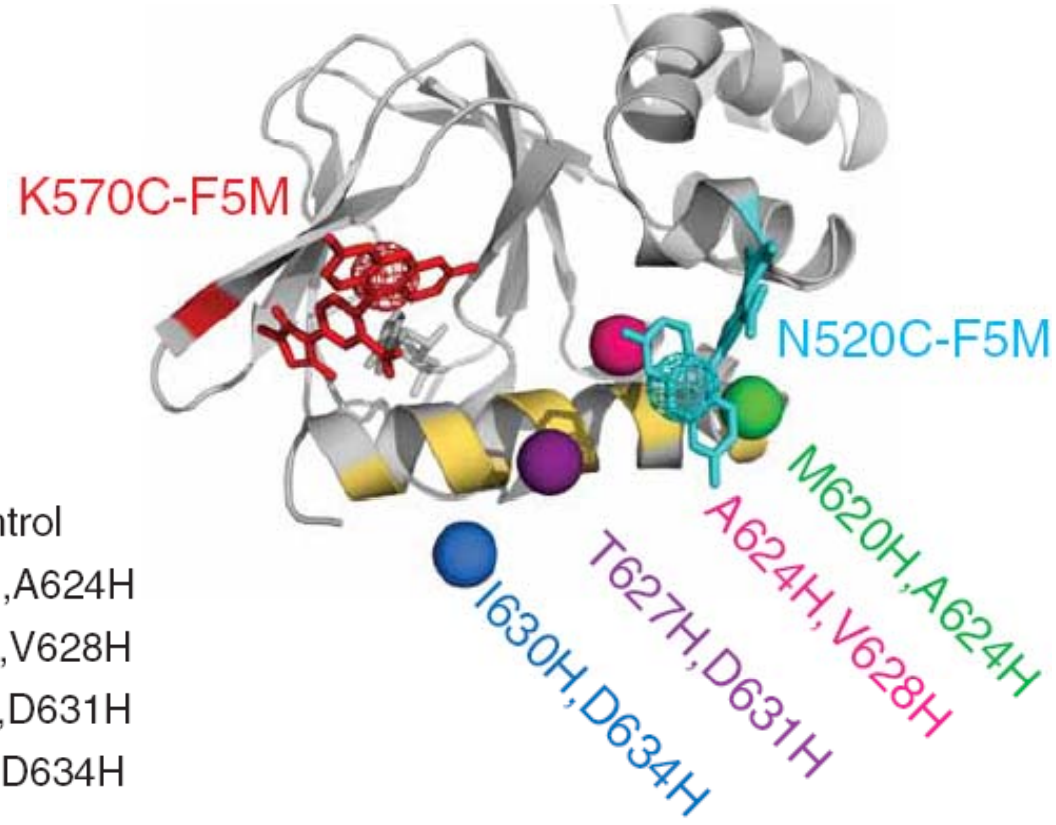


Holo vs. Apo by FRET



Holo vs. Apo by FRET





Model